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Award Number: DAMD17-01-1-0044

TITLE: A New Pathway of Chemotherapy Induced Apoptosis in a Prostate Cancer Cell Line

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REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
A New Pathway of Chemotherapy Induced Apoptosis in a Prostate Cancer Cell Line

5. FUNDING NUMBERS
DAMD17-01-1-0044

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

A current view is that cytotoxic stress, such as DNA damage, induces apoptosis by regulating permeability of mitochondria. Mitochondria sequester several proteins that, if released, kill by activating caspases, the proteases that disassemble the cell. Another way to activate caspases, which is used by cytokines, is to assemble receptor complexes that activate caspases directly, although the subsequent mitochondrial permeabilization accelerates cell disassembly by amplifying caspase activity. We found that cytotoxic stress causes activation of caspase-2 and that this caspase is required for permeabilization of mitochondria. Therefore, we argue that cytokine and stress-induced apoptosis act through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
prostate cancer, apoptosis, caspases	41
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Annual report for Year 01.

INTRODUCTION

To facilitate evaluation of this annual report, I would like to start with reviewing the purpose of the funded study and the approaches that we proposed to use to achieve our goals.

The purpose of the study. Most cells, including cancer cells resistant to available drugs, retain a suicide machinery that can kill within an hour through a process called apoptosis. A current view is that apoptosis is executed by a proteolytic cascade. The apoptotic cascade consists of caspases, a family of cysteine proteases, their co-factors and inhibitors. The cascade can be initiated at several points by activating so called initiator caspases. Each initiator caspase mediates a subset of cytotoxic signals. Therefore, the prerequisite to the understanding how a particular signal induces apoptosis is to identify the initiator caspase and its co-factors.

We found that apoptosis induced by chemotherapy in DU145, a prostate cancer cell line, required caspase activity, which was not surprizing. However, we failed to assign this activity to a known caspase, and in particular to the caspases that have been thought to mediate chemotherapy-induced apoptosis. This suggested that apoptosis in this cell line is mediated by an unidentified caspase that we called caspase-P. Because each known initiator caspase defines a distinct pathway of apoptosis, our hypothesis was that chemotherapy can induce apoptosis through a yet unidentified pathway that is defined by caspase-P. Because dissecting other pathways of apoptosis gave insights into the mechanisms of chemotherapy, provided markers for drug-sensitivity, and suggested new ideas for drug design, we proposed to identify and characterize caspase-P and its pathway.

The approaches. To identify caspase-P we proposed to exploit high affinity of caspases to p35, a viral caspase inhibitor. p35 inhibited chemotherapy-induced apoptosis in DU145 and was processed following drug treatment, which was consistent with the notion that p35 inhibits caspase-P. Because p35 forms highly stable complexes with inhibited caspases, we developed an approach to purify caspases by isolating the p35-caspase complexes. We also proposed alternative approaches, which included exploring putative caspases identified by the Human Genome Sequencing Project as caspase-P candidates.

BODY OF THE REPORT

What happened during the last year. Two events that occurred during the last year changed our approach to identification of caspase-P. The first event, which happened earlier than we expected, was the announcement that the sequence of the human genome has been largely completed. This development provided an opportunity to learn the total number of human caspases. The second development was the advent of RNA

interference (RNAi) as a tool to silence gene expression in mammalian cells, a technology that we could only dream of when we were submitting the proposal. We took advantage of these developments and decided to exploit small interfering RNA (siRNA) as a tool to systematically silence expression of caspases encoded in the human genome by RNAi.

The first targeted caspase-2 because we could not eliminate this caspase as a caspase-P candidate unambiguously. For technical reasons, we first tested the siRNA on human fibroblasts transformed with the adenoviral oncogene E1A. We unexpectedly found that this siRNA prevented apoptosis in the fibroblasts. Moreover, we established that caspase-2 was required for release from mitochondria of cytochrome c, a protein required for activation of caspase-9, a caspase that has been considered central for chemotherapy induced apoptosis. Our surprise was understandable considering a current view that caspase-2 is not a major caspase involved in drug-induced apoptosis and that cytochrome c release is caspase-independent. We found that caspase-2 is required for chemotherapy-induced apoptosis not only in our experimental system but also in at least some human cancer cells. Hence, our findings indicated that in response to chemotherapy mitochondria are amplifiers of caspase activity rather than initiators of caspase activation, as it has been widely believed. The study also raised the need to understand the regulation of caspase-2, which is practically unknown.

The details of the study are described in the attached manuscript that has been accepted for publication in *Science*. Besides identifying a conceptually new pathway of apoptosis, our atudy also demonstrated that RNAi can be successfully used to study human cancer cells. A description of the approach, which is attached, will be published in *Science's* Signal Transduction Knowledge Environment (STKE).

After "digressing" into studies of basic mechanisms of apoptosis, we used the tools that we developed to determine whether DU145 cells require caspase-2 to die and found that it is not. The success of the RNAi approach, however, encouraged us to systematically analyze the role of the remaining caspases, in particular ones that we could not unambiguously exclude in our preliminary investigation. This is where our research is now. Considering the efficiency of the RNAi approach, we are reasonably hopeful that we will identify caspase-P during this year and will begin studies of its regulation.

KEY RESEARCH ACCOMPLISHMENTS

We identified a central part of a major pathway of apoptosis.

REPORTABLE OUTCOMES

Lassus, P., Opitz-Araya, X., Lazebnik, Y. Requirement for caspase-2 in stress-induced apoptosis prior to mitochondrial permeabilization. *Science*, in press, 2002.

Lassus, P., Rodriguez, J., Lazebnik, Y. Confirming specificity of RNAi. *Science's STKE*, in press, 2002.

The results of the study were reported at the following scientific meetings and invited seminars.

Meetings:

The Servier International Conference "Understanding the molecular basis of cancer for therapeutic benefit", France
AACR Special Conference on Apoptosis and Cancer
EMBO Workshop on Nuclear Structure and Function, Prague

Seminars:

The National Institute of Child Health and Development
The New York University Cancer Center Seminar Series
Hutchison Research Center, Cambridge, UK
University of Vermont

CONCLUSIONS

Our systematic approach to chemotherapy-induced apoptosis helped us to advance the understanding of this process. The results of our study opened a new area of research and raised a likely possibility that a current model of chemotherapy-induced apoptosis, in which mitochondria play a central role, is inadequate.

THE GLOBAL WEEKLY OF RESEARCH
Science

3 July 2002

Dr. Yuri Lazebnik
Cold Spring Harbor Laboratory
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Cold Spring Harbor NY 11724

Ref: 1074721

Dear Dr. Lazebnik: Yuri

I am pleased to accept your paper "Requirement for Caspase-2 in stress-induced apoptosis and acts prior to mitochondrial permeabilization." for publication in *Science*. Your paper will now be prepared for publication by our copyediting department. They will edit your manuscript to improve clarity, to save space, and to conform to *Science* style. You will have an opportunity to approve these changes before publication. Our printer will send an email to you with instructions for viewing and approving your proofs online.

If you have not already sent your full contact information, including email address, of the author who will be responsible for checking galley proofs to Cara Tate at ctate@aaas.org and his/her schedule over the next several weeks, please do so immediately.

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The success of *Science* Magazine depends on your contributions. Thank you for submitting your best work to us.

Sincerely,

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Published by the American Association for the Advancement of Science

Confirming specificity of RNAi in mammalian cells.

Patrice Lassus, Joe Rodriguez, and Yuri Lazebnik

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ABSTRACT

RNA interference (RNAi) is a process of sequence-specific gene silencing. Recent advances in the understanding of RNAi have provided practical tools to silence gene expression in mammalian cells, which has opened novel possibilities to study functions of genes and proteins. To ensure that an observed effect of RNAi is due to silencing of the intended target, we suggest approaches to rescue the effect of RNAi by ectopically expressing the protein of interest. The approaches involve introducing silent mutations into the cDNA of the protein, and targeting RNAi to the untranslated regions of the gene.

INTRODUCTION

Preventing expression of a protein is an effective way of learning what this protein actually does or does not do. Until recently, this approach required a substantial amount of effort and was largely limited to animal models. The advent of RNA interference (RNAi) allows one to silence gene expression in a variety of cells, including human, which opened the possibilities that biologists only dreamed of. We will refer the reader to reviews (*1*) for detailed description of the RNAi technology and the underlying mechanisms. From the point of view of a practitioner, RNAi is a phenomenon that allows one to destroy an mRNA by introducing into a cell a double stranded RNA that is cognate to the target gene. Currently, the double stranded RNA can be introduced by transfection as a short synthetic or *in vitro* transcribed RNA duplex (siRNA) (*2*), or expressed from an appropriate vector either as a hairpin RNA or as two separate strands (*3,4*). Because the field is relatively new and develops rapidly, it is likely that by the time you read this protocol understanding of RNAi and the number of tools available for silencing genes will expand significantly.

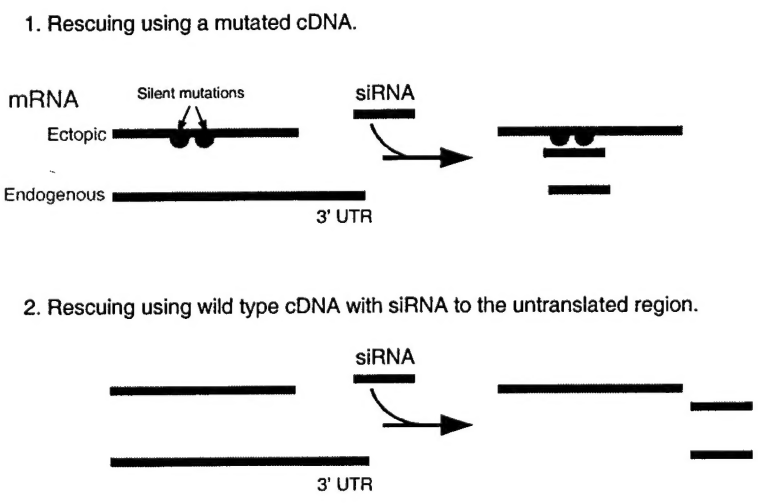

Figure 1. Rescuing the effect of RNAi by ectopic expression of proteins. See text for explanations.

Whatever the tool, you would want to confirm that the observed effect of RNAi is indeed due to silencing of the intended target. A usual way to test whether an effect is due to a deleted gene is to express the product of this gene ectopically. However, RNAi will silence expression of both endogenous and ectopic mRNA. We suggest two approaches to overcome this problem (Figure 1). One is to use a cDNA that has silent mutations in the region that is targeted by RNAi (Figure 1.1). These mutations prevent interaction of the RNAi machinery with the ectopic mRNA but do not affect destruction of the endogenous mRNA (*5*).

The second approach is to target RNAi to an untranslated region (UTR) of the gene of interest and to rescue the effect by expressing the wild type cDNA that does not contain the native UTR.

MATERIALS

Cell culture

DMEM with L-glutamine (Gibco)
Fetal Bovine Serum (FBS) (Gibco)
6-wells tissue culture dishes (Falcon)
10 cm tissue culture dishes (Falcon)
Phosphate-Buffered saline (PBS)
Trypsin EDTA (Gibco)

Retroviral transduction

Packaging cells (we use LinX available from Dr. Gergory Hannon (hannon@cshl.org))
Polybrene (Sigma)
Deionized water H_20
$CaCl_2$
Hepes
BES
NaCl
Na_2HPO_4
0.45 μ syringe filters (Millipore)
Siliconized 1.7 ml eppendorf tubes.

Transfection

OptiMEM (Gibco)
Oligofectamine Transfection reagent (Invitrogen)
Fugene (Roche)

Mutagenesis

QuikChange Site-Directed Mutagenesis Kit (Strategene)

EQUIPMENT

Tissue culture hood
Multi-block heater
Water bath
PCR machine
Micro centrifuge
A centrifuge that can centrifuge 6-well tissue culture plates.

RECIPES

Recipe 1: $CaCl_2$ solution

$CaCl_2$	2.5 M
Hepes	10 mM

Adjust pH to 5.5 with concentrated HCl

Recipe 2: 2xBBS

BES	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 M

Adjust pH to 6.95 with 10 M NaOH

Recipe 3: Polybrene solution

Polybrene	8 mg/ml in water.

INSTRUCTIONS

We will describe how to rescue the effect of RNAi using silencing by synthetic siRNA as an example. However, the principles of the rescue are likely to be applicable to other RNAi techniques. The instructions detail how to use siRNA in mammalian cells, and describe how to test whether the observed effect of siRNA is due to silencing of the intended target.

1. GENE SILENCING USING siRNA

Application of siRNA in mammalian cells involves the following steps: choosing the siRNA sequence, obtaining the siRNA from a supplier, transfection of the siRNAi, testing efficiency of gene silencing, and determining whether the silencing causes any changes in the experimental system.

1.1 Selection of siRNA sequences

We follow the guide provided by Tuschl's laboratory, who pioneered the siRNA approach (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html). A general rule is that the sequence should look like AA(19N)TT with a GC content of 30-70%. A computer program (http://www.ic.sunysb.edu/Stu/shilin/rnai.html) developed by Jack Lin in our laboratory uses this rule to selects potential siRNA and to find whether the selected sequences match cDNA sequences other than that of the intended target. In addition, it is important to consider that a guanine base in siRNA and the target RNA may pair not only with a cytosine but also with a uracil. In principle, both coding and untranslated regions (UTR) of and mRNA can be targeted. Targeting siRNA to UTR is not recommended because it is thought that regulatory protein complexes that bind to these regions will

interfere with the binding of the RNAi machinery. Yet, we found that five out of six siRNA that target the 3' UTR of six genes are very efficient and we suggest to use this approach if specificity of silencing is confirmed (see Fig. 2 for an example).

Although the available rules for siRNA selection are a reliable general guide, they do not guarantee that each of the selected siRNA will work. Therefore, you will need to decide how many siRNA to order, a choice that will be determined by your budget and the time that you can wait for the results. If you are on a tight budget, ordering siRNA one at a time may be an acceptable approach, as there is about 50% chance that the first siRNA you order will work well. If money is not an issue, we suggest ordering four siRNAs, two for the coding sequence, and two for the 3'UTR. The companies that provide synthetic siRNA and descriptions of their products can be found at the Tuschl's laboratory Web site. We have been using Dharmacon (http://www.dharmacon.com/) because of reliable service.

1.2 Preparation of siRNA for transfection

Dharmacon delivers siRNA in three options that vary in price. The most expensive option provides RNA duplexes that are ready for transfection. We usually use the less expensive option B, which provides purified single-strand lyophilized oligonucleotides that are ready to anneal. We resuspend the oligonucleotides in water provided by Dharmacon and anneal them as instructed by the manufacturer, which results in annealed siRNA at concentration of 20μM. The annealed siRNA is aliquoted and stored at –70°C.

1.3 Transfection of siRNA

Transfection of siRNA is the most critical factor that determines success of gene silencing. The transfection conditions will depend on the cells and the transfection reagents that you will use. The procedure that we will describe has been optimized for human fibroblasts transformed with the adenoviral oncogene E1A (*5*) and the Oligofectamine transfection reagent (Invitrogen). We found that this procedure is effective at least with some other cell lines.

All procedures are performed in a tissue culture hood at room temperature. PBS and media should be warmed to 37°C before use.

1. The day before transfection, plate cells in six-well plates to have them at 25-30% confluency (about 10^5 cells per well) at the time of transfection.

Note: Cell density is <u>really</u> important. Not enough cells per plate will result in a high background toxicity, too many cells will lower transfection efficiency. You will have to find the right cell density empirically.

2. Take two 1.5 ml siliconized eppendorf tubes for each siRNA that you are going to use.

Note: We suggest to use two siRNAs as controls: one that has no effect on your cells, for example targeting a gene that is not expressed in your cells, and another that has a

known and an easily tested effect, such as the siRNA to lamin A (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html), which is available commercially (http://www.dharmacon.com/).

3. Mix gently (pipet the solution or invert the tube, **NO** Vortexing) 10μl of siRNA duplex (20μM) with 200μl of OPTIMEM in the first tube.

4. In the second tube mix gently 10μl of oligofectamine with 50μl of OPTIMEM.

Repeat steps 3 and 4 for each siRNA.

5. Wait 5 minutes.

6. Combine contents of tube 1 and 2 and mix gently (by pipeting or inverting, **NO** Vortexing!!) to make the transfection mixture. Repeat this step for each siRNA.

7. Wait 20-25min.

8. Meanwhile, rinse cells with PBS and add 2 ml of fresh medium (DMEM-10% FBS without antibiotics) per well.

9. Add the transfection mixture drop by drop to the cells while gently agitating the plate and leave the cells overnight in an incubator.

***Note:** If the tranfection is toxic, you can rinse the cells after 6 hours.*

10. Replace the medium with 2 ml of DMEM-10% FBS per well.

11. Place cells back in the incubator and wait until the effect of siRNA, namely "disappearance" of the target protein from the cells, which may take several days and will depend on efficiency of siRNA and stability of the protein. We found that two days after siRNA transfection was optimal to decrease concentration of some proteins while others required four days of waiting. You have to determine the timing for your experimental system empirically.

1.4 Evaluating the efficiency of siRNA.

If the functional product of the gene is a protein, a direct way of measuring the effectiveness of siRNA is immunoblotting or immunofluorescence. If a specific antibody is not available, one possibility is to ectopically express the protein as a fusion with an epitope tag, such as HA or Myc, and use a commercial tag-specific antibody to determine protein expression. This approach is based on a reasonable assumption that expression of the endogenous protein will be as efficiently silenced as that of the ectopic. Please remember that in this case the sequence targeted by RNAi should be a part of the ectopically expressed mRNA.

2. RESCUE OF RNAi

2.1 Rescue by using a mutated cDNA.

This method uses ectopic expression of the target protein using a cDNA that contains silent mutations in the sequence complementary to siRNA (Figure 1.1). The protocol describes how to mutate a cDNA, how to express it in cells, and how to test whether this expression overcomes the effect of RNAi.

2.1.1 Mutagenesis

There are no rules to choose the bases to mutate except that the mutations must be silent. Consistent with published reports (*6*) we found that two mutations were sufficient to prevent the destruction of the mRNA by the siRNA, although introducing more mutations would probably only help. The mutations will be more efficient if placed together and as close as possible to the middle of the sequence complementary to siRNA, a region that is required for siRNA silencing (*6*). The mutations can be introduced by your favorite method. We follow the protocol provided with the QuikChange Site-Directed Mutagenesis Kit by Stratagene, which gives consistently reliable results (http://www.stratagene.com/manuals/200518.pdf). To aid the screening of mutants it is useful to combine silent mutagenesis with introduction of a restriction site. The Webcutter program which can be found at this Web site (http://www.ccsi.com/firstmarket/firstmarket/cutter/cut2.html) is helpful for this purpose.

2.1.2 Ectopic expression of proteins

Once you have generated a mutant cDNA you can attempt to rescue the phenotype obtained with the siRNA by expressing the cDNA in cells. To do so, you use transient transfection (2.1.2.2) or establish stable cell line. Our method of choice is retroviral transduction (2.1.2.1) because it provides a population of cells rather than single clones and allows one to obtain sufficient amount of cells within two weeks. Whatever the approach you use, you will need to confirm that siRNA results in silencing of expression of the endogenous but not of the ectopic gene. A convenient way of distinguishing the endogenous and ectopically expressed proteins is to express the ectopic protein as a fusion with an epitope tag. The tag may change the mobility of the protein in gel electrophoresis, which will separate the ectopic and endogenous proteins, and allow for detection of the ectopic protein independently of the endogenous (see Fig.2 for an example).

2.1.2.1 Retroviral transduction

The first step is to clone mutated as well as native cDNA into a retroviral vector containing a drug selection gene. Various systems for retroviral transduction are

available. We often use the MaRX system (*7*), which has been consistently efficient with our experimental systems.

Remember that even a retrovirus deficient in proliferation should be handled with caution. You may not want to express your favorite protein in yourself. Follow exactly the rules of your institution that govern the use of retroviruses and use common sense.

All solutions should be sterile.

Transfection of the retroviral vector:

1. Split the packaging line into a 6-well plate so that they are 70-80% confluent the next day. Plan the number of wells, considering that you would need to make cell lines that will express mutated and native cDNA. You will also need cells that are not trasfected by your retroviral vector (mock transfected) as a control for selection.

2. On the day of transfection change the media. Use 2 ml of media (DMEM-10% FBS) per well.

Wait at least 1 hr.

3. For each well to be transfected, aliquote 6 μg plasmid into 1.6 ml eppendorf tube, bring the volume up to 225 μl with ddH_20, and add 25 μl $CaCl_2$ solution (Recipe 1).

4. Add dropwise 250 μl 2X BBS (Recipe 2) while bubbling air through the solution with a Pasteur pipet connected to a Pipetaid. Add the resulting solution dropwise to a well with cells while swirling the plate.

5. Incubate at 37° overnight in an incubator with 3% CO_2.

6. Change media and incubate at 32° for 60 hr (2.5 days).

7. On the day before transduction, split the cells you want to transduce into a 6-well plate to have them 50-60% confluent at the time of transduction. Use 2 ml of media per well.

Transduction

8. Collect medium containing retrovirus from packaging line with a 3 ml syringe, attach a 0.45 μm filter and pass media through onto cells that you want to transduce. This will result in 4 ml of medium per well (2 ml of medium with the virus added to 2 ml of medium present in the well).

9. Add supernatant from untransfected packaging cells to one of the wells. These cells will be called mock-transduced.

10. Add 4 μl of 8 mg/ml polybrene (Recipe 3) to each well (final concentration of 8 μg/ml).

11. Spin plate in a tabletop centrifuge at 1700 rpm for 1 hr at room temperature.

12. Incubate at 32° overnight.

13. Change medium and incubate at 37° for 2 days (or until confluent).

14. Transfer cells from each well into a 10 cm tissue culture dish. The following day add selection drug, such as puromycin or hygromycin, which will be determined by the vector used. The appropriate drug concentration will be dependent on the cells used and must be determined empirically. Maintain cells in this medium until all mock-transduced cells die. The cells are ready to use. We suggest to test whether the protein is expressed and to make a stock of the cells before doing any experiments.

2.1.2.2 Transient transfection

If your favorite cell line can be transfected with a high efficiency (more than 80%), or if you can study your phenotype at the single cell level, for example by immunofluorescence, you may want to use transient transfection. An example of this approach is given in Fig. 2. You will have to determine empirically the right conditions (high efficiency with low toxicity) by varying cell density and transfection reagents. We routinely use Fugene from Roche or Lipofectamine 2000 from Invitrogen and follow instructions provided by the manufacturers. The transfection of your cDNA is done at least one day after transfection of the siRNA. The following typical procedure is performed at room temperature to transfect cells plated in 6-well tissue culture plates.

1. Label one 1.5 ml siliconized eppendorf tube for each plasmid.

2. Add 100μl of OptiMEM into each tube.

3. Add 5μl of Fugene and mix gently by pipetting

4. Add 2-3μg of plasmid, mix gently by pippeting.

5. Wait 20-25min.

6. Rinse cells with PBS and add 2 ml of fresh medium (DMEM-10% FBS without antibiotics) per well.

7. Add the transfection mixture drop by drop to the cells while gently agitating the plate and leave the cells overnight in an incubator.

8. Replace the medium with 2 ml of DMEM-10%FBS per well.

Note: If the tranfection is toxic, you can rinse the cells after 4-8 hours.

9. Place cells back in the incubator and wait one to two days until collecting the cells.

10. Test for protein expression by immunoblotting or im conditions munofluorescence. If it is expressed, use the cells, if not, optimize the or consider retroviral transduction.

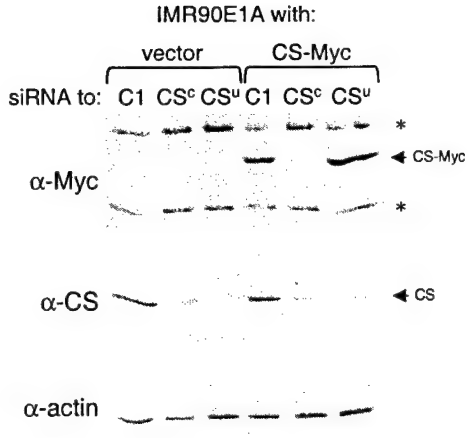

Figure 2. Rescue of RNAi silencing by ectopically expressing the target gene that is silenced by an siRNA to the gene's 3' UTR. This experiment was designed to test silencing of an abundant protein, citrate synthase (CS). Human fibroblasts transformed with adenovirus oncogene E1A were transfected with one of the following siRNA: an siRNA to caspase-1 (negative control), an siRNA to the coding region of the citrate synthase gene (CS^c) or an siRNA to 3' untraslated region (Cs^u) of the gene. One day later the cells were then transfected either with an empty vector (pMaRX) or the plasmid expressing a fusion of CS with a Myc epitope tag (pMaRX-CS-Myc). One day after transfection expression of CS and CS-Myc was analyzed by immunoblotting with antibodies to either CS or the tag. Note that expression of endogenous CS is repressed by siRNAs to the coding and untranslated region, while the expression of the ectopic protein is affected only by the siRNA to the coding region. The amount of CS-Myc in this experiment was only a fraction of the amount of endogenous CS. The concentration of β-actin and of the proteins cross-reacting with the antibody to the tag (indicated by asterisks) were used as controls for equivalent sample loading.

2.1.2 Does ectopic expression of a protein rescue the changes caused in your cells by RNAi?

To answer this question, do the following:

1. Treat the cells that ectopically express either native or mutated cDNA with siRNA (Instructions, section 1).

2. Determine protein expression by immunoblotting or by immunofluorescence. siRNA should silence expression of the endogenous protein and the protein that is expressed from the native but not from mutated cDNA. If this is the case, determine if the phenotype that you investigate is observed only in cells that do not express the protein. For an example see our study that describes silencing of caspase-2 (*5*).

2.2 Rescue by targeting 3' UTR

If you use an siRNA directed to the 3' UTR of your gene, you can rescue the effect of siRNA by ectopically expressing the protein using the wild type cDNA. You would need to make sure, however, that your expression plasmid does not contain the targeted sequence of the 3'UTR of your gene of interest. This rescue method is more practical if you have a collection of plasmids that express your favorite gene and its mutants, which would allow you to analyze these mutants for their function. The steps required to apply this approach are the same as described in section

2.1 except you would not need to mutate the cDNA of your favorite protein. An example of this approach is provided in Figure 2.

NOTES AND REMARKS.

Applications of RNAi in mammalian cells are likely to expand in scope and variety in the near future, which may likely affect how particular steps of this protocol should be implemented. We would appreciate comments and suggestions.

REFERENCES

1. G. J. Hannon. RNA interference. *Nature*. **418**, 244-51. (2002).

2. S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. **411**, 494-8. (2001).

3. T. R. Brummelkamp, R. Bernards and R. Agami. A system for stable expression of short interfering RNAs in mammalian cells. *Science*. **296**, 550-3. (2002).

4. P. J. Paddison, A. A. Caudy, E. Bernstein, G. J. Hannon and D. S. Conklin. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev*. **16**, 948-58. (2002).

5. P. Lassus, X. Opitz-Araya and Y. Lazebnik. Requirement for caspase-2 in stress-induced apoptosis prior to mitochondrial permeabilization. *Science*. **in press**, (2002).

6. S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel and T. Tuschl. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *Embo J*. **20**, 6877-88. (2001).

7. G. J. Hannon, P. Sun, A. Carnero, L. Y. Xie, R. Maestro, D. S. Conklin and D. Beach. MaRX: an approach to genetics in mammalian cells. *Science*. **283**, 1129-30 (1999).

Requirement for caspase-2 in stress-induced apoptosis prior to mitochondrial permeabilization.

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A current view is that cytotoxic stress, such as DNA damage, induces apoptosis by regulating permeability of mitochondria. Mitochondria sequester several proteins that, if released, kill by activating caspases, the proteases that disassemble the cell. Another way to activate caspases, which is used by cytokines, is to assemble receptor complexes that activate caspases directly, although the subsequent mitochondrial permeabilization accelerates cell disassembly by amplifying caspase activity. We found that cytotoxic stress causes activation of caspase-2 and that this caspase is required for permeabilization of mitochondria. Therefore, we argue that cytokine and stress-induced apoptosis act through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation.

Apoptosis is executed by caspases, a family of proteases that disassemble a cell (1, 2). The pathways leading to caspase activation vary with the cytotoxic stimulus. The stimuli that are collectively referred to as cytotoxic stress, such as DNA damage, activate caspases by initiating signaling pathways that converge on the Bcl-2 family of proteins (3). A balance between members of this family is thought to determine whether mitochondria remain intact or become permeabilized and release proteins that promote cell death (4). One of these released proteins is cytochrome c, which, in a complex with the cytoplasmic protein Apaf-1, activates caspase-9. Caspase-9, in turn, activates caspase-3, the protease that cleaves the majority of caspase substrates during apoptosis. Two other proteins, Smac (also called Diablo) and Htr2A (Omi), accelerate caspase activation and increase caspase activity by inactivating caspase inhibitors. Mitochondria also release apoptosis-inducing factor (AIF) and endonuclease G, which appear to kill independently of caspases. Therefore, mitochondria are thought to be a central regulatory element in stress-induced apoptosis (5).

Another way to activate caspases, used by cytokines, is to assemble receptor complexes that recruit caspase-8 or caspase-10 thereby inducing their autocatalytic processing (2). These caspases activate other caspases, including caspase-3, either directly by proteolytic processing, or indirectly, by cleaving a Bcl-2 family member Bid. A proteolytic fragment of Bid permeabilizes mitochondria thereby accelerating cell disassembly as described above. Hence, mitochondria in this pathway function as "amplifiers" of the caspase activity rather than as central regulators of apoptosis.

This model of apoptosis was consistent with studies of oncogene-dependent apoptosis, a phenomenon that may provide clues how to kill cancer cells selectively (6). By comparing normal human fibroblasts (IMR90) with fibroblasts transformed with the adenoviral oncogene E1A (IMR90E1A), we found that this oncogene sensitizes cells to chemotherapeutic drugs by facilitating activation of caspase-9 (7). E1A appears to achieve this effect by promoting activation of Bax, a pro-apoptotic Bcl-2 protein that can permeabilize mitochondria, and by repressing a yet unidentified inhibitor of this permeabilization (8). These observations were in agreement with the model that Bcl-2 proteins control caspase activation by regulating mitochondrial permeability. Contrary to this view, we report that mitochondrial permeability is controlled by a caspase which is activated earlier in the pathway that links DNA damage and cell disassembly.

We used small interfering RNA (siRNA) to silence expression of various proteins by RNA interference (RNAi) in IMR90E1A cells (9). An siRNA to caspase-2, a caspase that has been implicated in apoptosis but whose exact function and regulation remain unknown (10-12), efficiently and specifically silenced caspase-2 expression (Fig. 1A). Several other siRNAs (13) had no effect on expression of caspase-2. Only siRNA to Apaf-1 repressed expression of Apaf-1, and none of the siRNAs used affected expression of caspase-9 (Fig. 1A). The siRNA to Apaf-1 prevented apoptosis induced by all three DNA damaging agents (etoposide, cisplatin, and UV light) that we used (Fig. 1B). To our surprise, however, inhibiting expression of caspase-2 prevented apoptosis as efficiently as did inhibiting expression of Apaf-1. This effect could not be attributed to manipulations required to introduce siRNAs into cells, because siRNA to caspase-1 had

no effect on apoptosis (Fig. 1B). Therefore, we conclude that either caspase-2 is required for apoptosis or the caspase-2 siRNA interfered with expression of other proteins.

To test whether apoptosis indeed required caspase-2, we attempted to restore sensitivity to cytotoxic agents by expressing caspase-2 ectopically. To prevent destruction of the ectopic caspase-2 mRNA by the caspase-2 siRNA, we introduced two silent mutations into the region of the caspase-2 cDNA that is complementary to the siRNA (14). Consistent with reported high specificity of siRNA (15), expression of the endogenous caspase-2 or of the ectopic caspase-2 from the wild type cDNA was efficiently silenced, whereas the expression of caspase-2 from the mutated cDNA (caspase-2si) was not (Fig. 2A). Expression of caspase-2si restored the sensitivity of cells to etoposide (Fig. 2B), indicating that the effect of the siRNA could be explained by repression of caspase-2. Expression of catalytically inactive caspase-2si (in which catalytic cysteine was mutated to serine) (14) did not rescue the effect of the siRNA, indicating that proteolytic activity of caspase-2 is required to mediate apoptosis (Figure 3). The absence of caspase-2 did not affect the rate of apoptosis induced by TNF-alpha (Fig. S1), indicating that caspase-2 is not required for receptor-mediated apoptosis.

After exposure of cells lacking caspase-2 to etoposide, cytochrome c remained in mitochondria (Fig. 4A and Fig. S3), as did Smac (Fig. 4B and Fig. S3). Both proteins were released in the absence of Apaf-1, indicating that experimental manipulations required to introduce siRNA into cells do not prevent mitochondria permeabilization. Caspase-2 was proteolytically processed even if processing of caspases 9, 3 and 7 was

prevented by siRNA to Apaf-1, indicating that caspase-2 is activated before or independently of the other three caspases (Fig. S2). Hence, caspase-2 is required to permeabilize mitochondria.

The earliest detectable change in the apoptotic machinery after DNA damage may be the translocation of the cytoplasmic Bcl-2 family member Bax to mitochondria (16). Etoposide induced Bax translocation in cells transfected with the siRNA to Apaf-1. However, Bax remained in the cytoplasm in cells transfected with the siRNA to caspase-2 (Fig. 4C and Fig. S3). Thus, caspase-2 is required to translocate Bax to mitochondria in this experimental system.

Hence, as previously suggested (17), our results imply that stress-induced apoptosis can be executed in a pathway that is conceptually similar to that of cytokine-induced apoptosis, in that each pathway begins with activation of a caspase that uses mitochondria to amplify the total caspase activity in the cell.

We expanded our study to five human tumor cell lines that can be efficiently transfected, are sensitive to cytotoxic agents, and are widely used in cancer research. In the lung adenocarcinoma A549 and the osteosarcoma U2OS, caspase-2 and Apaf-1 were required to produce morphological features of apoptosis (Fig. 5 A and B), and, in both cell lines, caspase-2 was required for cytochrome c release (Fig. 5 C). In contrast, breast cancer cell line MCF-7 did not require caspase-2 for release of cytochrome c, perhaps because cytochrome c release is caspase-independent in these cells or because other caspases are

involved (13). The results obtained with cervix adenocarcinoma HeLa and colorectal carcinoma HCT-116 cells were inconclusive due to poor viability of these cells when transfected with siRNA.

Our findings have several implications (18). Although strong evidence indicates that a failure of apoptosis contributes to cancer progression in experimental systems, the evidence is much weaker for such a relationship in human cancers (19). Evidence for the latter has been primarily gathered by correlating tumor properties with deficiencies in the apoptotic machinery. Our findings imply that a critical part of a major apoptotic pathway is yet to be considered by such studies. Indeed, survival of cancer cells might be enhanced by any changes that prevent caspase-2 activation. How this activation is regulated is unknown, which indicates that even basic pathways of apoptosis are yet to be sufficiently explored to allow the efficient modulation of apoptosis to a therapeutic end. This study also highlights truly novel opportunities provided by RNAi to study cancer biology without relying only on observations made with genetically modified mice.

FIGURE LEGENDS.

Fig. 1. Requirement of caspase-2 for apoptosis induced by several cytotoxic agents. **(A)** IMR90E1A cells were transfected with siRNA to Apaf-1 (A-1), caspase-2 (C2), caspase-1 (C1) (as a control for the effect of transfection), or left untransfected (14). After culturing cells for 2 days a portion of the cells was used to determine amounts of caspase-2 and Apaf-1 by immunoblotting (14). **(B)** The remaining cells were treated with indicated cytotoxic agents (closed bars) or left untreated (open bars). The final concentration of the drugs in the medium was 50 μM for etoposide and 20 μM for cisplatin. UV was used at 10 mJ/cm^2. After 18 hours of treatment both adherent and floating cells were collected, fixed with 4% paraformaldehyde and stained with DAPI to reveal chromatin structure. Cells with condensed chromatin were scored as apoptotic.

Fig. 2. Restored sensitivity of cells to apoptosis after ectopic expression of caspase-2. **(A)** cDNA encoding caspase-2 (C2) or caspase-2 in which two silent mutations prevented interaction between siRNA and the caspase-2 mRNA (C2si) (14) were ectopically expressed in IMR90E1A cells by retroviral transduction (14). The cell lines were transfected with siRNA to caspase-2 or caspase-1 or left untransfected. Two days later the amount of caspase-2 in the cell lines was determined by immunoblotting (14). The ectopic protein contains a Myc epitope tag that allows distinction of endogenous and ectopic caspase-2 by a difference in electrophoretic mobility. The blot was re-probed with an antibody to beta-actin to indicate the relative amount of total protein applied in each lane (14). **(B)** The cells transfected with siRNA as described in (A) were treated

with 50 μM etoposide (closed bars) or left untreated (open bars) and the rate of apoptosis was determined (Fig. 1).

Fig.3. Requirement of caspase-2 activity to rescue stress-induced apoptosis. **(A)** caspase-2si (C2si, also see Fig.2) or caspase-2si in which the catalytic cysteine was mutated into a serine (C2siCys) (14) were expressed in IMR90E1A cells by retroviral transduction (14). The cell lines were transfected with siRNA to caspase-2 to or to caspase-1 (14) and expression of endogenous an ectopic caspase-2 was determined by immunoblotting. **(B).** The cells transfected with siRNA to caspase-2 as described in (A) or left untransfected were treated with 50 μM etoposide for 18 hours and scored for apoptosis (Fig. 1).

Fig. 4. Requirement of caspase-2 for release of cytochrome c and Smac from mitochondria, and for translocation of Bax from the cytoplasm to mitochondria. IMR90E1A cells were transfected with siRNA to either Apaf-1 or caspase-2. After two days the cells were either treated with 50 μM etoposide or left untreated. Eighteen hours after treatment the cells were fixed, cytochrome c, Smac, and Bax were visualized by imunofluorescence (14) (Fig. S4, S5, and S6), and the fraction of cells in which cytochrome c or Smac was released from mitochondria (A, B) or Bax translocated from the cytoplasm to mitochondria (C) was determined by counting 400 to 500 cells for each cell population. The cells were counterstained with DAPI to visualize the nuclei.

Fig. 5. Requirement of caspase-2 for apoptosis and cytochrome c release in human tumor cell lines. **(A)**. Indicated cell lines were treated with siRNAs to caspase-2 or Apaf-1, or

caspase-1 (14) and expression of Apaf-1 and caspase-2 was determined by immunoblotting with antibodies to these proteins (14). The blot shown was exposed after incubation with both antibodies. **(B)** Cells transfected with siRNA to caspase-1, APAF-1, and caspase-2 were treated with cisplatin (40 μM) for 20 hours, collected and scored for apoptosis by observing chromatin condensation (Fig. 1). **(C)** Cytochrome c release in the cells treated as described in (B) was visualized by immunofluorescence and the fraction of cells with released cyt c was determined by counting about 500 cells.

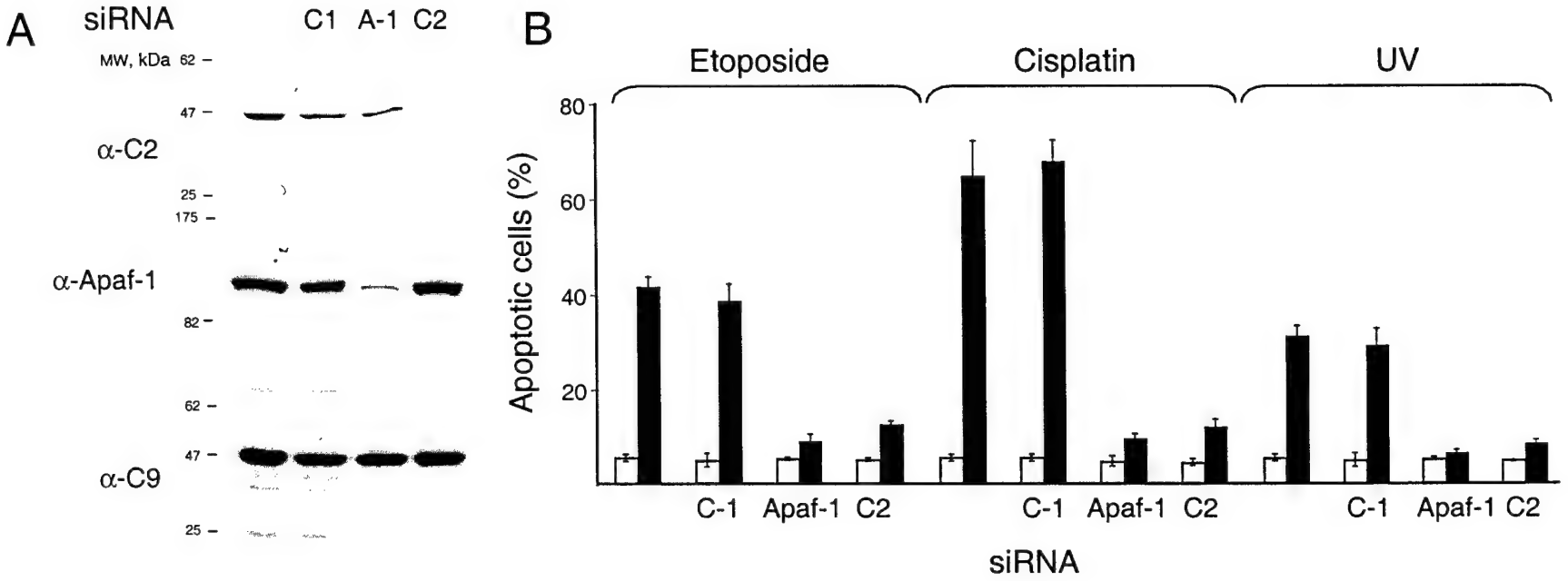
REFERENCES.

1. N. A. Thornberry, Y. Lazebnik, *Science* **281**, 1312-6 (1998).
2. M. O. Hengartner, *Nature* **407**, 770-6. (2000).
3. A. Gross, J. M. McDonnell, S. J. Korsmeyer, *Genes Dev* **13**, 1899-911. (1999).
4. D. C. Huang, A. Strasser, *Cell* **103**, 839-42. (2000).
5. X. Wang, *Genes Dev* **15**, 2922-33. (2001).
6. G. Evan, T. Littlewood, *Science* **281**, 1317-22 (1998).
7. H. O. Fearnhead *et al.*, *Proc Natl Acad Sci U S A* **95**, 13664-9 (1998).
8. D. M. Duelli, Y. A. Lazebnik, *Nat Cell Biol* **2**, 859-862 (2000).
9. S. M. Elbashir *et al.*, *Nature* **411**, 494-8. (2001).
10. S. Kumar, M. Kinoshita, M. Noda, N. G. Copeland, N. A. Jenkins, *Genes Dev* **8**, 1613-26. (1994).
11. L. Wang, M. Miura, L. Bergeron, H. Zhu, J. Yuan, *Cell* **78**, 739-50. (1994).
12. L. Bergeron *et al.*, *Genes Dev* **12**, 1304-14. (1998).
13. P. Lassus, Y. Lazebnik, *Unpublished.*
14. Materials and methods are available as supporting material on Science Online.
15. S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, *Embo J* **20**, 6877-88. (2001).
16. A. Nechushtan, C. L. Smith, Y. T. Hsu, R. J. Youle, *Embo J* **18**, 2330-41 (1999).
17. G. Hausmann *et al.*, *J Cell Biol* **149**, 623-34. (2000).
18. Supporting Online Material, Supporting text.
19. I. Herr, K. M. Debatin, *Blood* **98**, 2603-14. (2001).

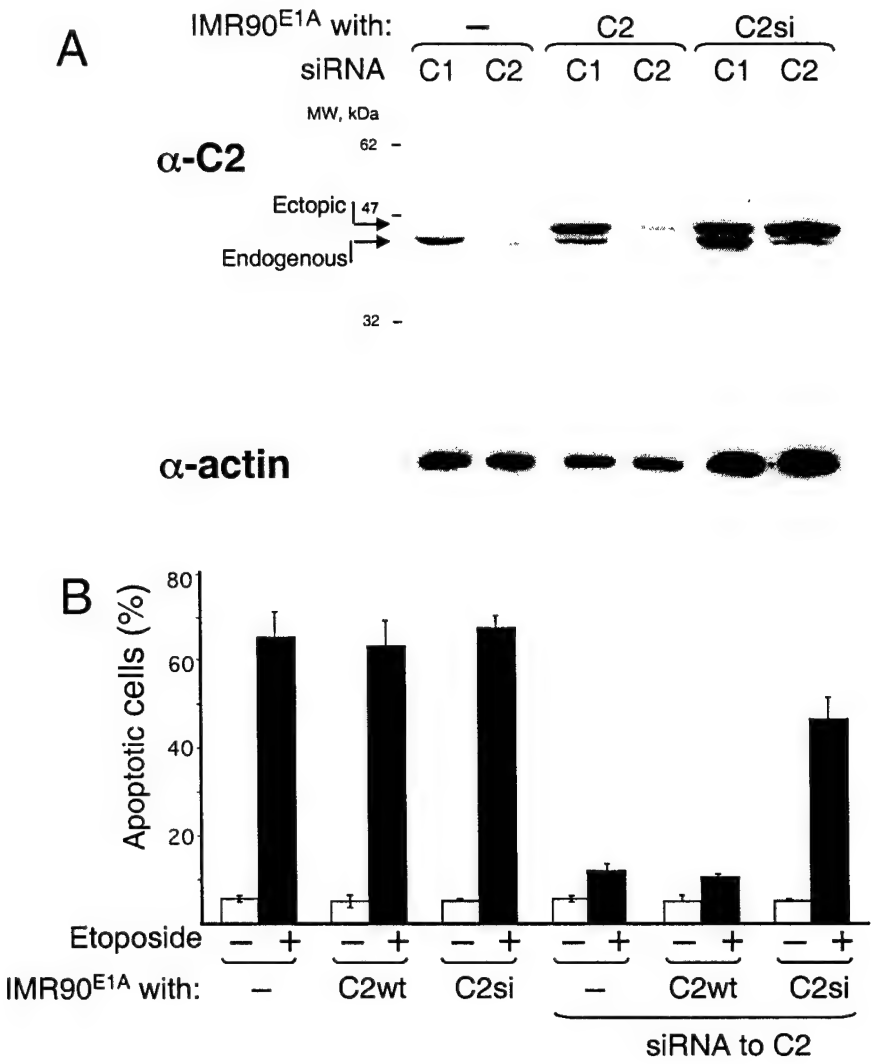
ACKNOWLEDGEMENTS.

We would like to thank Vanessa Marsden and Andreas Strasser (Walter and Elisa Hall Institute) for enjoyable interactions and for sharing unpublished data, and the members of the Lazebnik laboratory, Scott Kaufmann, Greg Hannon, and Scott Lowe for helpful

discussions. This study was supported by US Army Prostate Cancer Program grant DAMD17-01-1-0044, and the National Institutes of Health grant CA-13106-31 to YL.



Lassus et al. Figure 1.



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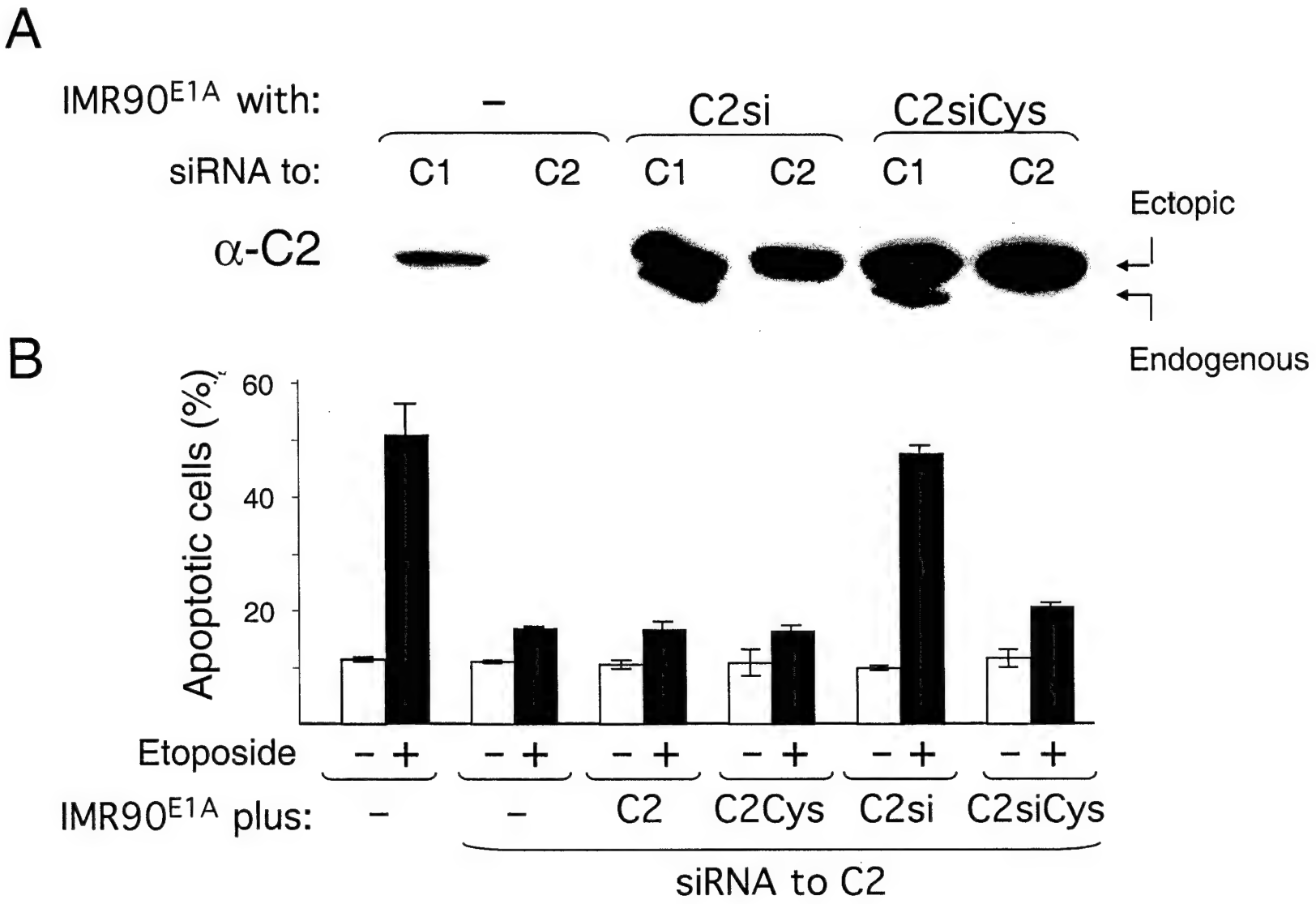
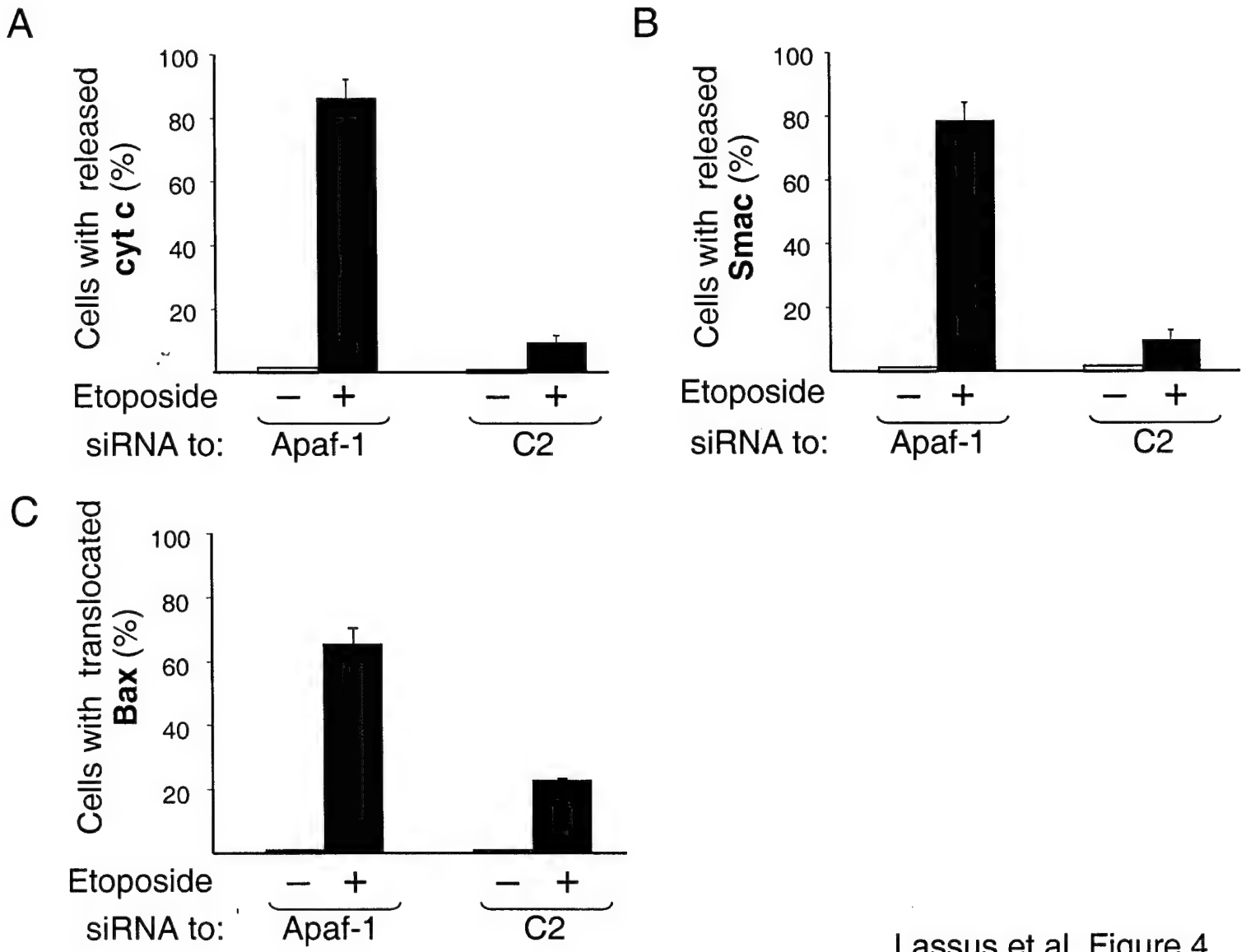
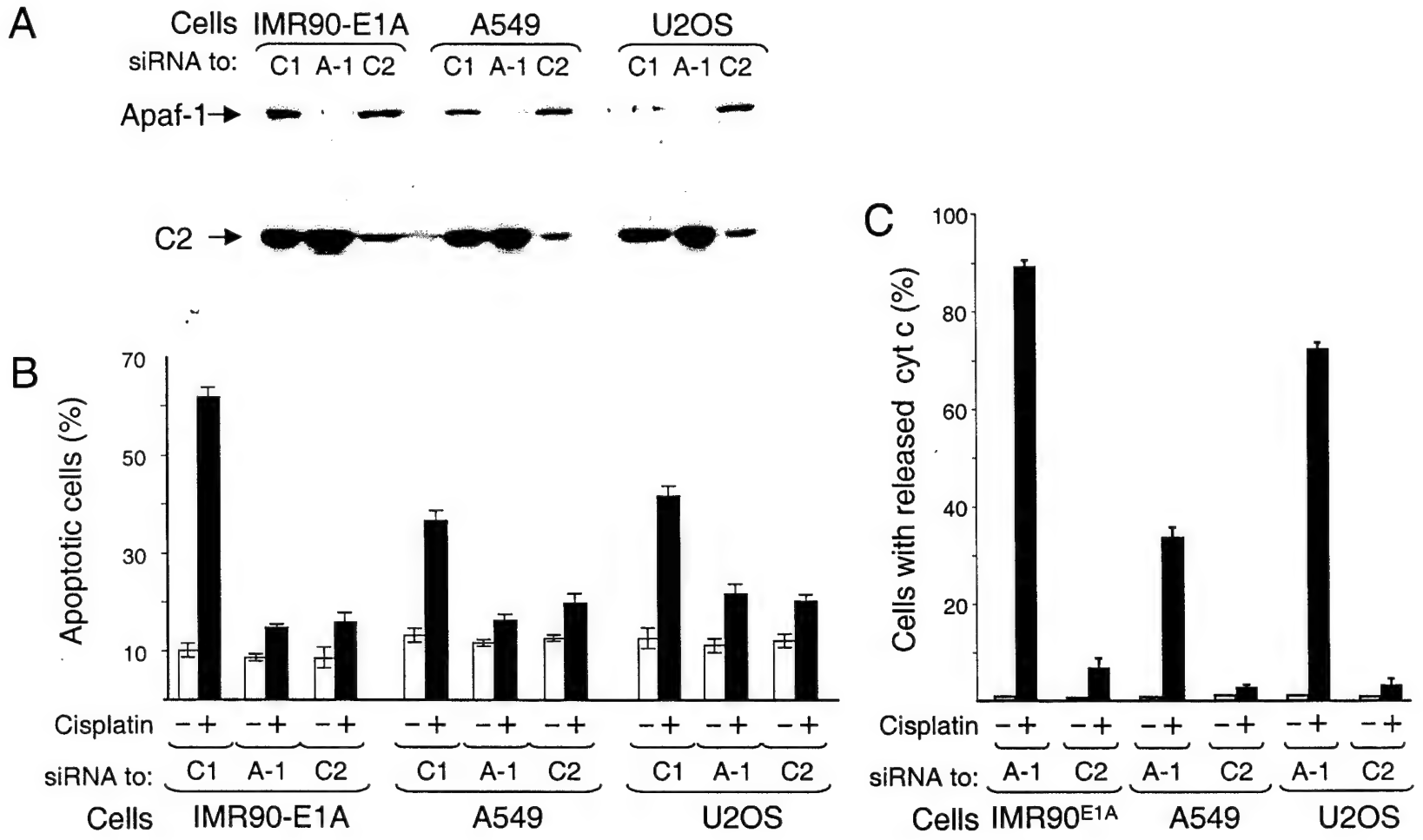


Lassus et al. Figure 3 A, B.



Lassus et al. Figure 4



Lassus et al. Figure 5.

SUPPORTING ONLINE MATERIAL.

Materials and methods.

Preparation of siRNA. Sense and anti-sense oligonucleotides corresponding to the following cDNA sequences were purchased from Dharmacon: AAGGGGCACAGGCATGCCAAA (nucleotides 191-211) for caspase-1, AATTGGTGCACTTTTACGTGA (nucleotides 978-998) for Apaf-1; and AAACAGCTGTTGTTGAGCGAA (nucleotides 94-114) for caspase-2. The sense and anti-sense oligonucleotides were annealed following manufacturer's protocol to generate the double stranded siRNAs at the final concentration of 20 μM.

Mutagenesis. To introduce silent mutations into the region of the caspase-2 cDNA that is cognate to the siRNA used in this study, the nucleotides GC in the positions 99-100 were mutated into AT by using Quick Change kit (Stratagene) and the mutagenesis confirmed by sequencing. To obtain a catalytically inactive caspase-2, the same approach was used to mutate the codon encoding the catalytic cysteine (Cys 303) into one encoding a serine.

Transfection of siRNA. Cells were cultured in 6-well plastic plates in 2 ml of DMEM medium complemented with 10% FBS and transfected at 40% of confluency by adding 10 µl of oligofectamine (Invitrogen) and 10 µl of 20 µM siRNAs (final concentration 100 nM). Cells were rinsed with the medium after 16 hrs of incubation and then maintained in culture for additional 32 hrs before analysis. The efficiency of transfection varied between 80% and 90%.

Generation of cell lines by retroviral transduction. cDNAs for caspase-2 and its mutants were cloned into a MarxIV-puro retroviral gene transfer vector (S1) to be expressed as fusions with a carboxy-terminal Myc epitope tag. Retroviruses were produced by transfection into LinX-A packaging cells (kindly provided by Gregory Hannon). Medium from transfected LinX-A cells supplemented with 8 mg/ml Polybrene was diluted 1:2 with fresh medium and was added to plates of IMR90-E1A cells. Plates

were centrifuged at 1,000 3 g for 1 hour and then were incubated for 12–18 hours at 32°C. Medium then was replaced after 2 days, and infected cells were selected for 4 days by using puromycin to use in the study.

Antibodies. All antibodies used in this study were monoclonal. The antibodies to caspase-9 and Apaf-1 have been described previously (S2). The antibody to caspase-2 was a kind gift from Drs. David Huang and Andreas Strasser (WEHI, Australia), the antibody to Bid was a kind gift from Dr Junying Yuan (Harvard Medical School). The antibody to Smac was developed by the authors at Cold Spring Harbor Laboratory by immunizing mice with the recombinant protein that was kindly provided by Dr. Alnemri (Kimmel Cancer Center). The specificity of the antibody was confirmed using cells in which expression of Smac was silenced by RNAi. The antibody to cytochrome C was purchased from Pharmingen and the antibody to beta-actin from Sigma. Immunofluorescence was performed as previously described (S3).

Supporting text.

This study has several implications in addition to those mentioned in the main text. One is related to the basic design of apoptotic pathways. Genetic studies in the nematode *C. elegans* provided an outline of the apoptotic machinery. In this organism, CED-4, an activator of the caspase CED-3, is sequestered by a Bcl-2 family member CED-9. EGL-1, another Bcl-2 family member, releases CED-4 from CED-9 thus triggering CED-3 activation and cell death. Our finding that caspase-2 is required early in apoptosis provides an experimental model to test the hypothesis that in mammals, as in the nematode, the Bcl-2 proteins control apoptosis by directly sequestering molecules required for activation of a caspase (S6).

Another implication is related to the current model of how mitochondria permeability is regulated during apoptosis. It is thought that cytotoxic stress, such as DNA damage, activates Bax and other pro-apoptotic proteins of the Bcl-2 family, which permeabilize mitochondria thus releasing factors required for caspase activation. Our observations

argue that at least in some cell types the roles of Bax and caspase activation are reversed, in that a caspase activity is required for Bax translocation. This conceptual change may help to understand how Bax is activated. For example, caspase-2 may process Bax or its regulators.

Our study also raises several questions. Arguably, a major reason why caspase-2 and its regulation have been hardly studied is that caspase-2-deficient mice have only a few abnormalities (S7). Neurons of these mice are resistant to beta-amyloid (S8), the mice have supernumerary oocytes, these oocytes are resistant to the anti-cancer drug doxorubicin, and B-lymphocytes of these mice are resistant to apoptosis induced by granzyme B and perforin, the proteins used by cytotoxic lymphocytes to kill target cells (S7). Yet, we find that caspase-2 is required for a fundamental pathway of apoptosis whose deficiency has severe consequences in mice. For example, mice deficient in Apaf-1 die shortly after birth because of severe anatomical abnormalities (S9). These observations raise a question as to why a deficiency in caspase-2 does not have a similar outcome.

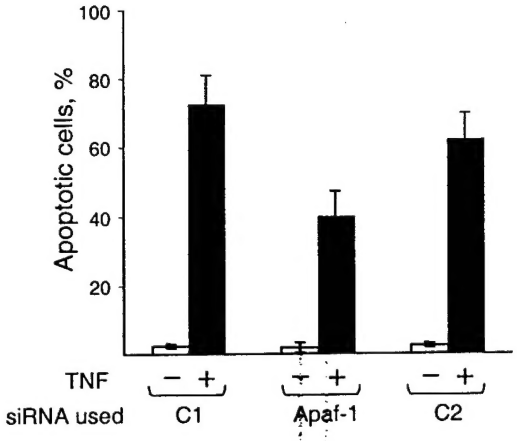
One possibility is that other caspases can substitute for caspase-2. This would be consistent with the observation that although oocytes of caspase-2 deficient mice are resistant to chemotherapy, the blastocysts from these mice are sensitive, suggesting that shortly after fertlization caspase-2 becomes either redundant or that the deficiency is rescued by compensatory caspase activation (S10). A compensatory activation would not be surprising considering that compensation of a gene deficiency is often observed in knockout mice (S11). For example, although a majority of Apaf-1 deficient mice have severe abnormalities, about 5% of the population are viable and have no apparent defects except of male sterility (S12).

The apparent discrepancy between the results obtained using genetically engineered mice and human tumor cells highlights truly novel opportunities provided by RNAi for studies of cancer biology. Until the advent of RNAi, the mechanisms of apoptosis in human cancer cells had to be extrapolated from observations made with genetically modified

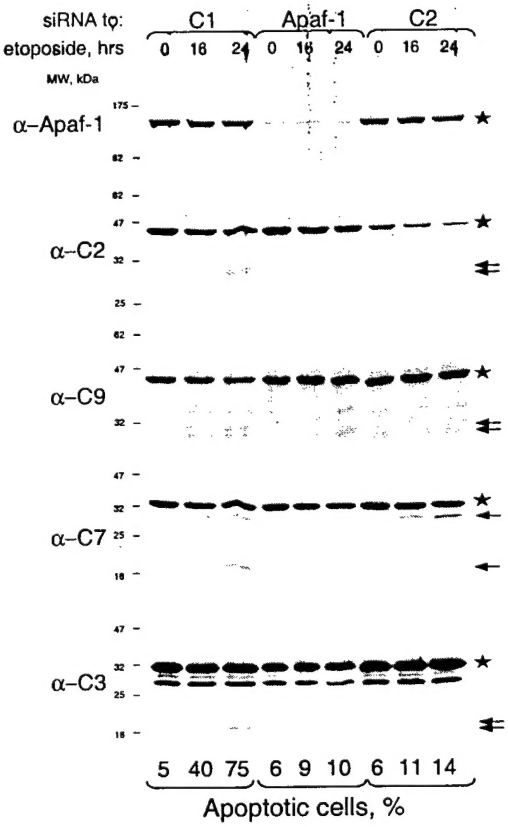
mice. Such extrapolations were bound to create misconceptions, one of which appears to be the underestimation of the caspase-2 role in apoptosis.

Another question raised by our findings is why previous studies found no role for caspases in cytochrome c release in stress-induced apoptosis (S13, S14). How indeed our results can be reconciled with the previous reports? An answer might be in the differences between the approaches. The previous studies based their conclusions largely, if not exclusively, on the observation that a wide-range peptide caspase inhibitor ZVAD-fmk fails to prevent cytochrome c release. However, caspase-2 is several orders of magnitude more resistant to ZVAD-fmk than other caspases (S15), which implies that the requirement for this caspase could not be reliably determined by using this reagent. Such unusual resistance of caspase-2 to peptide inhibitors should also be considered in determining whether cell death requires a caspase activity, as such determination often relies solely on the ability of ZVAD-fmk to prevent apoptosis.

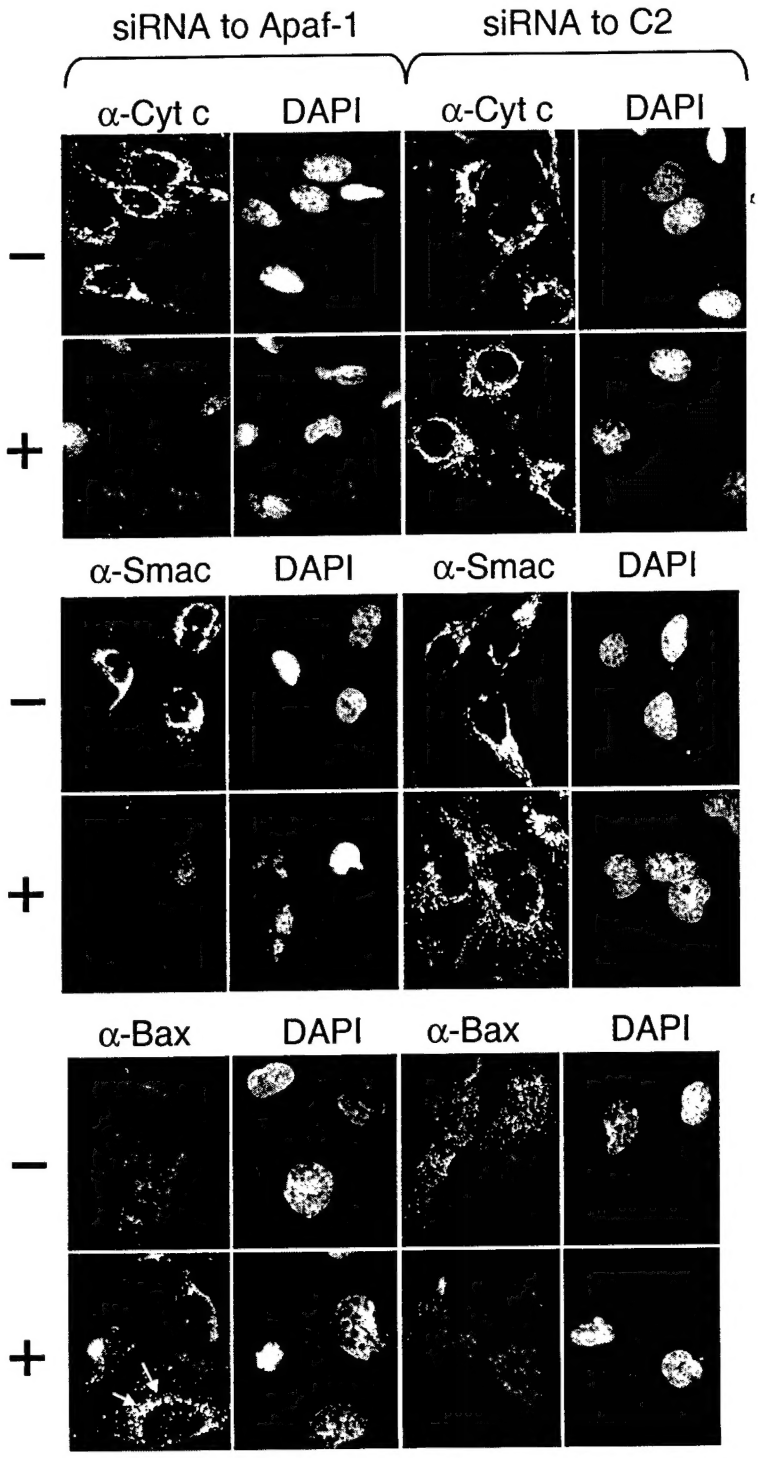
Supporting figures.



Supporting Fig. 1. siRNA to caspase-2 does not affect the rate of TNF-alpha-induced apoptosis. IMR90E1A cells were transfected with indicated siRNAs and then treated with 35 ng/ml of TNF-alpha in a combination with 5 μg/ml of cycloheximide. Cells were collected after 10 hours of treatment, fixed, stained with DAPI and cells with condensed chromatin were scored as apoptotic as described in Fig 1 of the main report.



Supporting Fig 2. Caspase-2 is proteolytically processed even if expression of Apaf-1 is repressed. IMR90E1A cells were transfected with the indicated siRNAs (Supporting Online Materials, Methods), treated with 50μM etoposide and collected at the indicated times. The cells were lysed and the expression of the indicated protein was determined by immunoblotting (Supporting Online Materials, Methods). The positions of caspase precursors are indicated by stars, the positions of the processed caspases by arrows. Note that caspase-2 has two processing sites between the large and the small subunit (S4), which is consistent with the observed two polypeptides generated by processing. Interestingly, one of the polypeptides is predominant in the absence of Apaf-1, suggesting that, perhaps, like in caspase-9, one of the sites is processed autocatalytically while another by the downstream caspases. Caspase processing in extracts from cells transfected with caspase-2 siRNA could be explained by the fractions of untransfected cells.



Supporting Fig. 3.

Requirement of caspase-2 for release of cytochrome c and Smac from mitochondria and for translocation of Bax to mitochondria from the cytoplasm. IMR90E1A cells were transfected with siRNA to either Apaf-1 or caspase-2. After two days the cells were either treated with 50 μM etoposide (+) or left untreated (–). Eighteen hours after treatment the cells were fixed, and cytochrome c, Smac, and Bax were visualized by imunofluorescence as described in Methods (Supporting Online Materials). The cells were counterstained with DAPI to visualize the nuclei. The fraction of cells in which cytochrome c or Smac were released or Bax translocated was determined by counting 400 to 500 cells for each cell population and is presented in Fig. 4 of the main report. The white arrows at the bottom panel indicate translocated Bax.

Supporting references.

S1. G. J. Hannon *et al.*, *Science* **283**, 1129-30 (1999).

S2. J. Rodriguez, Y. Lazebnik, *Genes Dev* **13**, 3179-3184 (1999).

S3. H. O. Fearnhead *et al.*, *Proc Natl Acad Sci U S A* **95**, 13664-9 (1998).

S4. H. Li *et al.*, *J Biol Chem* **272**, 21010-7. (1997).

S5. D. M. Duelli, Y. A. Lazebnik, *Nat Cell Biol* **2**, 859-862 (2000).

S6. G. Hausmann *et al.*, *J Cell Biol* **149**, 623-34. (2000).

S7. L. Bergeron *et al.*, *Genes Dev* **12**, 1304-14. (1998).

S8. C. M. Troy *et al.*, *J Neurosci* **20**, 1386-92. (2000).

S9. H. Yoshida *et al.*, *Cell* **94**, 739-50. (1998).

S10. T. S. Zheng *et al.*, *Nat Med* **6**, 1241-7 (2000).

S11. R. Gerlai, *Behav Brain Res* **125**, 13-21. (2001).

S12. N. Honarpour *et al.*, *Dev Biol* **218**, 248-58. (2000).

S13. R. M. Kluck, E. BossyWetzel, D. R. Green, D. D. Newmeyer, *Science* **275**, 1132-1136 (1997).

S14. E. BossyWetzel, D. D. Newmeyer, D. R. Green, *EMBO J* **17**, 37-49 (1998).

S15. P. G. Ekert, J. Silke, D. L. Vaux, *Cell Death Differ* **6**, 1081-1086 (1999).